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Determinants of recognition of ligands binding to benzodiazepine receptor/GABA$_A$ receptors initiating sedation

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Abstract

Complementary behavioral and computational studies of 21 structurally diverse, gamma-amino butyric acid (GABA)$_A$ benzodiazepine receptor ligands that influence spontaneous locomotor activity have been performed in this work. This behavioral endpoint is a well-accepted indicator of sedation particularly for GABA$_A$/benzodiazepine receptor ligands. The goal of the work presented here is the identification and assessment of the minimum requirements for ligand recognition of GABA$_A$/benzodiazepine receptors leading to activity at the sedation endpoint embedded in a common 3D pharmacophore for recognition. Using the experimental results, together with a systematic computational procedure developed in our laboratory, a five-component 3D pharmacophore for recognition of the GABA$_A$ receptor subtypes associated with the sedative behavioral response has been developed consisting of: two proton-accepting moieties, a hydrophobic region, a ring with polar moieties and an aromatic ring in a common geometric arrangement in all ligands having an effect at the sedation endpoint. To provide further evidence that the 3D pharmacophore developed embodied common requirements for receptor recognition, a pharmacophore analysis was performed for agonists, inverse agonists and antagonists separately. Each of the resulting pharmacophores contained the same five moieties at comparable distances to those found for the pharmacophore generated using all of them together. This result confirms that this pharmacophore constitutes a recognition pharmacophore representing required features in the overlapping portion of their binding sites. The reliability of this 3D pharmacophore was then assessed in several ways. First, it was determined that ligands that had no effect at the sedation endpoint did not comply with the pharmacophore requirements. Second, four benzodiazepine receptor ligands known to have an effect at the sedation endpoint, but not used in the pharmacophore development were found to satisfy the requirements of this pharmacophore. Third, the geometric and chemical requirements embedded in this pharmacophore were used to search 3D databases resulting in the identification of benzodiazepine receptor ligands known to affect sedation, but not included in the pharmacophore development. Finally, a 3D-quantitative structure analysis procedure (QSAR) model was developed based upon the ligands in the training set superimposed at their sedation pharmacophore points. The 3D-QSAR model shows good predictivity for binding of these ligands to receptor subtypes containing $\alpha 1$ but not $\alpha 5$ subunits. The pharmacophore developed for the sedation endpoint thus provides a predictive binding model for diverse ligand binding to $\alpha 1$ containing receptor subtypes.

Keywords: GABA$_A$/benzodiazepine receptor; Sedation; 3D pharmacophore

1. Introduction

Benzodiazepines ligands are structurally diverse compounds that bind to a specific binding site on the gamma-amino butyric acid (GABA)$_A$ receptor, a pentameric chloride ion channel. These benzodiazepine receptor ligands allosterically modulate the effect of the endogenous neurotransmitter, GABA on chloride flux through the GABA$_A$ receptor ion channel. Benzodiazepine receptor ligands can either enhance, diminish or have no influence on GABA activity and have thus been categorized as agonists, inverse agonists and antagonists, respectively. Benzodiazepine receptor ligands influence a variety of behavioral endpoints including anxiolysis, sedation, hyperphagia, anticonvulsion and hyperthermia.

Progress in understanding the pharmacology of this receptor system has been complicated by the well-recognized heterogeneity of the GABA$_A$ receptor. It is now clear that there is more than one type of functional GABA$_A$

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receptor in virtually every region of the brain (Laurie et al., 1992; McKernan and Whiting, 1996; Wisden et al., 1992). Cloning and sequencing of this receptor has revealed that the pentameric $GABA_A$ ion channel consists of various combinations of subunits from eight distinct subunit classes: ($\alpha 1-6$, $\beta 1-4$, $\gamma 1-4$, δ, $\rho 1-3$, ε, π, θ) (Burt and Kamatchi, 1991; Olsen and Tobin, 1990; Bonnert et al., 1999; Olsen and DeLorey, 1999). Although in principle, these subunits can combine to form an essentially infinite number of functional $GABA_A$ receptors, estimates suggest far fewer distinct functional combinations in vivo (Whiting, 1999).

There has been long standing uncertainty in the number and subunit composition of the $GABA_A$ receptors associated with many behavioral activities. Nevertheless, studies of different combinations of subunits in transfected cell systems have identified important general characteristics of functional $GABA_A$ receptors. For example, with few exceptions, the co-expression of α, β, and γ subunits in transfected cell systems are required to mimic the pharmacological properties of native $GABA_A$ receptors (Prichett et al., 1989). However, studies of structurally diverse benzodiazepine receptor ligands on transfected cells containing various α subunits in combination with $\beta 2$, $\gamma 2$ subunits, have revealed little or no selective binding. The absence of selective ligands makes it difficult, from receptor binding studies alone, to associate a particular benzodiazepine receptor/$GABA_A$ receptor subtype with a given behavior. In addition, some $GABA_A$ receptors may have two distinct α subunits present in some tissues (Araujo et al., 1996).

A very recent study by Rudolph et al. (1999) provides the first direct evidence for the possibility of associating particular receptor subtypes with specific behavioral endpoints. The $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$ subunits have a conserved histidine (H) in position 101 and bind classical benzodiazepine ligands, such as diazepam, with comparable affinities. In contrast, $\alpha 4$ and $\alpha 6$ subunits have a conserved arginine (R) in this position and are diazepam insensitive. In that study, a H101R-mutated $\alpha 1$ subunit was introduced into mice and their pharmacological characterization in response to diazepam evaluated. The findings in this paper indicate the H101R mutation in the $\alpha 1$ subunit impairs the sedative, amnesia and anticonvulsant effects of diazepam administered to the transgenic mice, hence implicating H101 in these behavioral endpoints. These results imply that the $\alpha 1$ subunit is associated with at least these three behavioral endpoints.

Work by Dunn et al. (1999) has revealed that mutations at H101 can have significant effects on binding affinities as well as altering the characteristic agonist, inverse agonist or antagonistic response of RO15-4513 and RO15-1788 as determined by electrophysiological responses. These results reveal the sensitivity of binding affinities and specific types of activation to a single site-specific mutation in α subunits of the $GABA_A$ benzodiazepine receptor, hence making the task of relating receptor types to behavioral endpoints even more challenging.

In the joint experimental and computational study reported here, structurally diverse benzodiazepine receptor ligands were assessed for their effect on spontaneous locomotor activity. The effect of a drug on spontaneous locomotor activity in rodents has commonly been used as a measure of its sedative potential (Pain et al., 1999; Uvnas-Moberg et al., 1994).

Competitive binding studies indicate that, with the possible exception of low affinity sites, the binding of agonists, inverse agonists and antagonists are mutually exclusive (Dunn et al., 1999; Hunkeler et al., 1981; Mohler et al., 1981). This result implies that the binding site of agonists, inverse agonists and antagonists are at least partially overlapping. The overlapping binding sites, of sedation agonists, inverse agonists and antagonists must have a similar 3D recognition pharmacophore containing recognition moieties in their overlapping binding regions given that they all have significant binding affinities. These ligands, of course, have additional stereochemical features since they are structurally diverse, bind to and activate benzodiazepine receptors associated with other behavioral endpoints, as well as differentially activate the receptor associated the sedation activity. However, the analysis of the common 3D distance relationship specifying the arrangement of recognition elements common to the binding sites of sedation agonists, inverse agonists and antagonists, comprise a set of minimal requirements for recognition of their overlapping binding regions. A 3D pharmacophore descriptive of this overlapping binding regions should comprise elements constituting a significant portion of the binding affinities of the three classes of ligands at the receptor associated with the sedation endpoint. The determination of such recognition pharmacophores from such data set should then result in the identification of the minimal molecular recognition determinants shared by these three categories of ligands.

The goal of the work presented here is the identification and assessment of these minimum requirements, embedded in a common 3D pharmacophore for recognition. To this end the 21 structurally diverse ligands found to be active at this endpoint were used in an in house program, called MOLMOD to generate such 3D pharmacophore for recognition of receptors leading to activity at the sedation endpoint. This program does not require a template and makes no a priori assumptions of the biologically active conformation. Instead it requires the conformational library of each ligand as input together with candidate moieties selected as possible components of this pharmacophore. The program then determines if, for at least one possible conformer of each ligand, there is a common geometric arrangement of some of these candidate moieties. The reliability of this 3D pharmacophore was then critically assessed in several ways. First, ligands that had no effect at the sedation endpoint were analyzed to verify that they did not comply with the pharmacophore requirements. Second, four additional benzodiazepine receptor ligands known to have an effect at the sedation endpoint, but not used in the pharmacophore development were analyzed to verify that they satisfied the requirements of this pharmacophore. Third, the geometric and chemical requirements embedded in this pharmacophore were used to search 3D databases to verify that such a search results in the identification of benzodiazepine receptor ligands known to affect sedation, but not included in the pharmacophore development. Finally, a 3D-quantitative structure analysis procedure (QSAR) model was developed based upon the ligands in the training set superimposed at their sedation pharmacophore points in order to verify its correlation with the binding affinities of benzodiazepine receptors containing α1 subunits.

2. Materials and methods

2.1. Experimental methods

2.1.1. Animals

Male C57Bl/6 mice were obtained from Charles Rivers (Holister, CA) at 6 weeks of age. Mice were maintained on a normal 12 h light/12 h dark cycle (lights on 700–1900 h) with free access to food and water. Tests were conducted during the light phase between 1300 and 1700 h with a 30-min acclimation period in the testing room prior to drug or vehicle administration.

2.1.2. Drugs

The benzodiazepine receptor ligands tested were RO15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate), RO16-6028 (*t*-butyl(*S*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*][1,4]benzodiazepine-1-carboxylate), RO-23-1590 (2-(*p*-chloro phenyl)-4-(4-*N*-ethylamide piperazinyl) quinoline), RO15-1788 (8-fluoro-3-carboxy-5,6-dihydro-5-methyl-6-oxo-414-imidazo[1,5-*a*]1,4 benzodiazepine), RO41-7812 (7-chloro-4,5-dihydro-3-(3-hydroxy-1-propynyl)-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one), RO42-8773 (7-chloro-3-[3-(cyclopropylmethoxy)-1-propynyl]-4,5-dihydro-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepine-6-one)) from Hoffman–LaRoche, Nutley, NJ); CGS8216 (2-phenylpyrazolo[4,3-*c*]quinolin-3(5*H*)-one), CGS9896 (2-*p*-chlorophenylpyrazolo[4,3-*c*]quinolin-3(5*H*)-one), CGS9895 (2,5-dihydro-2-(4-methylphenyl)-3*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one 2-(4-methoxy-phenyl)-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one)-(Ciba–Geigy, Summit, NJ); RU31719 (Roussel Uclaf, Romainville, France) and AHR 11797 (A.H. Robbins, Richmond, VA); MFB 43C (1,3 dihydro-5-(2-thienyl)-2*H*-1,4-benzodiazepin-2-one) provided by Dr. Mark F. Beatty at the Univ. of Bath, U.K; compound #42 (ethyl 8-trimethylsilyl-2-accetyl-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*]1,4 benzodiazepine 1-carboxylate, compound #47 (ethyl 8-trimethylsilyl-2-accetyl-12, 12*a*-dihydro-9-oxo-9*H*, 11*H*-azeto[2,1-*c*]imidazo[1,5-*a*] 1,4 benzodiazepine 1-carboxylate) and RY10: (ethyl 8-ethyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate) were generously provided by Dr. James Cook at the Univ. of Wisconsin. All drugs were suspended in vehicle (0.9% saline containing 0.2% tween 80).

The data for abercanil (isopropyl-6-benzyloxy-4-methoxymethyl-β-carboline-3-carboxylate), AHR 14749 (1*N*,*N*′dimethyl-amido-imidazo[2,3*a*]-2-chloropyridine) and zolpidem (*N*,*N*,6-trimethyl-2-(4-methylphenyl)imidazo [1,2-*a*]pyridine-3-acetamide hemitartrate] are the only compounds whose results are shown in Table 1 and Fig. 1 which have not been re-examined using the protocols documented below but are as described in previously published work (Davies et al., 1994; Chen et al., 1996).

2.1.3. Spontaneous locomotive activity

Thirty minutes after receiving an intraperitoneal (i.p.) injection of drug or vehicle, mice were placed individually into a clear plastic monitoring chamber measuring 72 × 32 × 32 cm (San Diego Instruments, San Diego, CA). Spontaneous locomotor activity was measured via seven sets of photoelectric sensors evenly spaced along the length of the monitoring chamber, 4 cm above the floor of the chamber. Interruption of the photocell beams were recorded automatically and stored on computer. Both ambulatory and total locomotor activities were recorded. The monitoring period lasted for 10 min. Each set of mice was divided into two groups of four with one group receiving vehicle and the other the test drug. One week later the groups were switched, with the original vehicle group receiving drug and the original drug group receiving the vehicle.

Compounds were tested over the dose range 0.3–30 mg/kg. The minimal dose able to elicit a statistically significant difference from that of the vehicle by spontaneous locomotor activity assessment is reported in Table 1. Full dose response curves for a drug effect on spontaneous locomotor activity have been previously determined in our laboratory for 17 of the 21 compounds used in this study (data not shown). Spontaneous locomotor activity measurements were repeated on these 17 compounds to assure self-consistency with data collected on the newly added compounds (RY10, compound #42, compound #47 and RO15-4513).

Compounds that did not significantly affect spontaneous locomotor activity either by reducing activity (agonist) or enhancing activity (inverse agonist), as compared to vehicle, at a concentration of ≤ 30 mg/kg were also tested for antagonism. Characterization of antagonistic properties was accomplished by simultaneous i.p. injection of both the putative antagonist and the agonist flunitrazepam (5 mg/kg) and measuring effects on locomotor activity 30

Table 1
Spontaneous locomotor activity observed for 17 structurally diverse $GABA_A$ /benzodiazepine receptor ligands

Compound	Dose (mg/kg)	Activity[a]
Saline	NA	159 ± 22
Vehicle	NA	182 ± 7
CGS9895	10	52 ± 8^b (agonist)
CGS9896	30	123 ± 27
CGS8216	50	285 ± 30^b (inverse agonist)
RO15-1788	30	246 ± 33
RO15-4513	30	286 ± 19^b (inverse agonist)
RO16-6028	100	96 ± 34
RO23-1590	10	86 ± 16^c (agonist)
RO41-7812	10	309 ± 22^b (inverse agonist)
RO42-8773	30	282 ± 22^c (inverse agonist)
MFB 43C	30	111 ± 17
RY10	30	310 ± 27^b (inverse agonist)
Compound #42	30	226 ± 38
Compound #47	30	248 ± 20
RU31719	30	93 ± 17^c (agonist)
AHR 11797	30	105 ± 17
U 78875	30	121 ± 24
Flunitrazepam	5	56 ± 14^b (agonist)
Antagonism[d]		Drug + flu (5 mg/kg)
RO15-1788	10	157 ± 13^e (antagonist)
AHR 11797	30	36 ± 5 (no antagonism)
MFB 43C	30	28 ± 11 (no antagonism)
RO16-6028	10	219 ± 68^e (antagonist)
Compound #42	30	258 ± 63^e (antagonist)
Compound #47	10	128 ± 25^e (antagonist)
U 78875	30	269 ± 35^e (antagonist)

[a] Values are expressed as the mean ± SEM of the total number of times a mouse interrupted the photosensor beams during 10 min monitoring sessions. The experiments were conducted using eight mice to a group with the exception of the vehicle group which contained 12 mice. Differences in activity were determined by one-way ANOVA with significance reported between vehicle and each drug by post hoc comparisons using Dunnett's multiple comparison test.

[b] $p < 0.01$.

[c] $p < 0.05$.

[d] Differences between treatment groups in the antagonism study (vehicle, flunitrazepam, and simultaneous administration of drug with flunitrazepam) were determined by one-way ANOVA with post hoc comparisons using Bonferroni's multiple comparison test.

[e] Significantly different from flunitrazepam alone but not different from vehicle. Vehicle is 0.9% saline + 0.2% Tween 80. Flu = flunitrazepam.

min after injection. Results were compared to both the effects of flunitrazepam alone and the vehicle control.

Statistical evaluation of the locomotor activity data was assessed for multiple comparisons using one-way analysis of variance (ANOVA) with significance reported between vehicle control and each drug by post hoc comparisons using Dunnett's multiple comparison test. Differences between treatment groups in the assessment of antagonism (vehicle control, flunitrazepam, and simultaneous administration of benzodiazepine receptor ligand and flunitrazepam) were analyzed by one-way ANOVA with post hoc comparisons using Bonferrroni's multiple comparison test. GraphPad PRISM 2.01 (GraphPad Software, San Diego, CA) was used to perform the above analyses.

2.2. Computational methods

2.2.1. Construction of structures of benzodiazepine receptor ligands

Initial structures of the 21 benzodiazepine receptor ligands included in this study were constructed in MSI Quanta (MSI-Quanta/Biosym, San Diego, CA). All forcefield parameters used were from the Quanta/CHARMM (Chemistry at Harvard Macromolecular Mechanics) forcefield. The net atomic charges used, based on parameterization in this program, were also consistent with the fact that all of the compounds are neutral. The initial structures were then energy minimized using 200 steps of steepest descents followed by 2000–3000 steps of conjugate gradients or until the rms changes in the gradient were smaller than 0.01 Å. A long 90-Å potential truncation was used to minimize effects on structure due to potential truncation via a switching function.

2.2.2. Calculation of conformational libraries of the benzodiazepine receptor ligands

The method used for pharmacophore development requires the generation of a conformational library for each compound. These conformational libraries were generated with an assumed dielectric constant of 80 and a long potential function truncation distance at 90 Å in order to minimize truncation effects. The value of $\varepsilon = 80$ chosen is based on an assumption that the accessible benzodiazepine receptor ligand conformational pool is developed in a polar environment prior to binding to the GABA receptor or that the GABA binding sites are themselves partially exposed to a polar environment.

The method chosen for calculation of the conformational libraries depended on the number of rotatable bonds in the molecule. In all cases, the same Quanta/CHARMM field was used for the ligands. For benzodiazepine receptor ligands with less than five rotatable bonds, a simple nested rotation (grid search) procedure was used, as embodied in Quanta/CHARMM, employing incremental rotations about each rotatable bond and energy minimization with the torsion angles active in the grid search constrained to their grid values. For one compound in this study, abercanil, a hybrid genetic algorithm/energy minimization method was used, as described below.

The protocols adopted in preparation of conformational libraries by grid search procedures were based upon a few simple principals: (1) it is desirable to use fine grids for torsion angles affecting the position and orientation of hydrogen bonding moieties in the ligands, (2) the torsion angles affecting the position of large hydrophobic groups may be sampled more coarsely, commensurate with the overall goal of (3) keeping the total number of conformers in each ligand conformational library to less than several thousand. Given that hydrogen-bonding atoms in a ligand

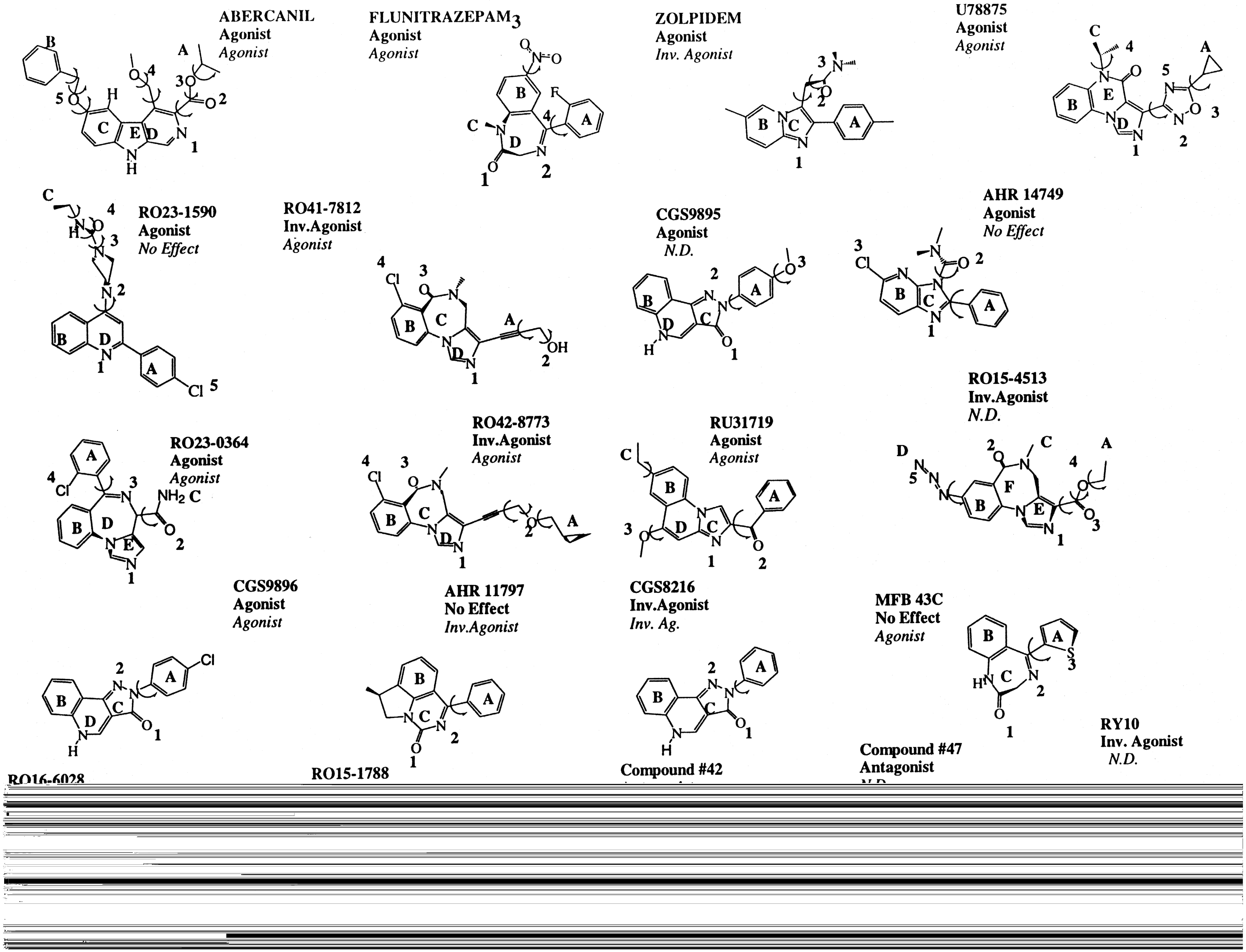

Fig. 1. Structures of the benzodiazepine receptor ligands used in this study. The name of the compound, its sedation (in bold) and anxiolytic response (in italics) are indicated. The lettering and numbering schemes indicate moieties used in pharmacophore development as described in methods, results and discussion. Torsion angles explored in the conformational library development for each of the compounds is indicated by arrows.

3 kcal/mol and a distance test tolerance of 1.3 Å. This pharmacophore for recognition of receptors leading to activity at the sedation endpoint is shown schematically in Fig. 2 together with the distance between each of the five components that make up the pharmacophore as well as their uncertainties. As shown in this figure, the five moieties that comprise this pharmacophore are two proton acceptors, one hydrophobic group, another that also contains the LUMO and an aromatic ring with polar moieties. These five moieties that comprise the pharmacophore are identified in Table 5 in a lettering/numbering scheme shown in Fig. 1 and the other property tables. Since agonists, antagonists and inverse agonists must all have significant affinity at these receptors, these common interactions found using all three types of ligands represent the minimum requirements for recognition by them of these receptors. These requirements are part of the overlapping regions of all three types of ligands.

This deduction is supported by an additional computational effort reported here. Specifically, a pharmacophore analysis using MOLMOD was performed for agonists, inverse agonists and antagonists separately. The systematic search in MOLMOD resulted in a pharmacophore for each class: agonists, inverse agonists and antagonists taken separately. Fig. 3 shows a bar graph comparing the corresponding distances between common moieties in the three classes of ligands deduced from this procedure, with those of the 3D pharmacophore deduced using all of them together. The results indicate that, within computed variability these distances are the same. This similarity found is a confirmation that the 3D pharmacophore generated shown in Fig. 2 contains the minimum requirements for ligand recognition of receptors leading to activity at the sedation endpoint. These requirements are part of the overlapping region of these ligands. Fig. 4 shows the overlap of $GABA_A$/benzodiazepine receptors ligands associated with sedation superimposing the five moieties of their recognition pharmacophore. This figure showing the distribution of the proton donating receptor points (complementary to ligand-based proton acceptors), in green, on either side of the long axis of the molecule defined by a hydrophobic group and the LUMO ring at opposite ends of each compound.

3.6. Assessment of the reliability of the 3D pharmacophore for recognition of receptors leading to activity at the sedation endpoint

Five assessments of the reliability of the sedation recognition pharmacophore were made. The first was to determine if the two compounds, AHR 11797 and MFB 43C, found to have no effect satisfied the requirements of the

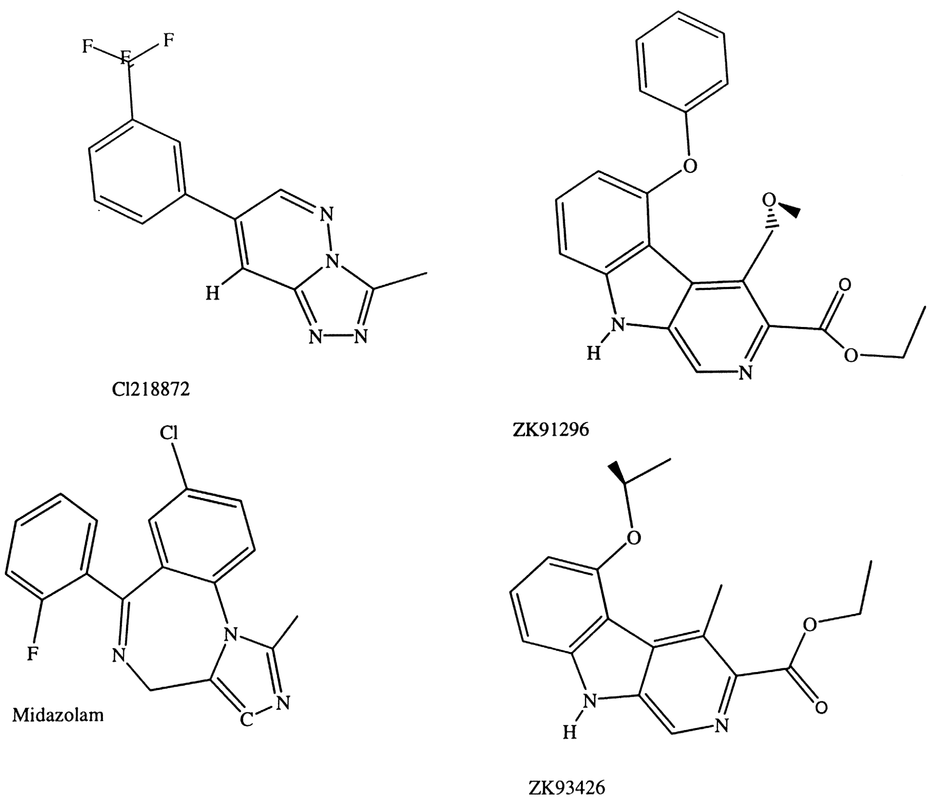

Fig. 5. Four ligands known to bind to receptor subtypes associated with sedation Cl218872 (Gardner and James, 1987), ZK91246/weak agonist (Pellow and File, 1986), midazolam (agonist) and ZK93426 (antagonist/weak inv. ag. (Duka et al., 1988)).

pharmacophore. No pharmacophore was found when these no-effect compounds were included in the data set even using twice the distance tolerance used to obtain the pharmacophore (of 1.3 Å). Moreover, both of these no-effect compounds were found to have two proton acceptor moieties near one another and on the same, rather than the opposite, side of the long axis of the ligand defined by the hydrophobic and aromatic ring containing LUMO. Both the lack of compliance of these compounds with the 3D pharmacophore developed and the qualitatively different arrangement of these two groups than found for the 19 compounds that had some activity at this endpoint are consistent with their lack of activity and provide a validation of the pharmacophore.

The second type of assessment of the pharmacophore was to determine the extent to which four additional benzodiazepine receptor ligands, not included in its development and known to be active at this endpoint, complied with the pharmacophore requirements. The four structurally diverse compounds chosen, known to bind to receptor subtypes associated with sedation Cl218872 (Gardner and James, 1987), ZK91246/weak agonist (Pellow and File, 1986), midazolam (agonist) and ZK93426 (antagonist/weak invag. (Duka et al., 1988)) are shown in Fig. 5 together with their known activity at the sedation endpoint. Conformational libraries were generated for these compounds and used as input to the MOLMOD program. The results obtained indicated that all four compounds satisfied the requirements of the 3D pharmacophore developed for sedation.

The third assessment made of the reliability of the 3D pharmacophore for the sedation endpoint was to use it to search 3D databases for compounds that satisfied its requirements, using the NCI, Chappman–Hall and Cambridge Crystallographic Databases. Fig. 6 shows eight compounds found from these database searches that are either known benzodiazepine receptor ligands with activity at the sedation endpoint or closely related analogs of such

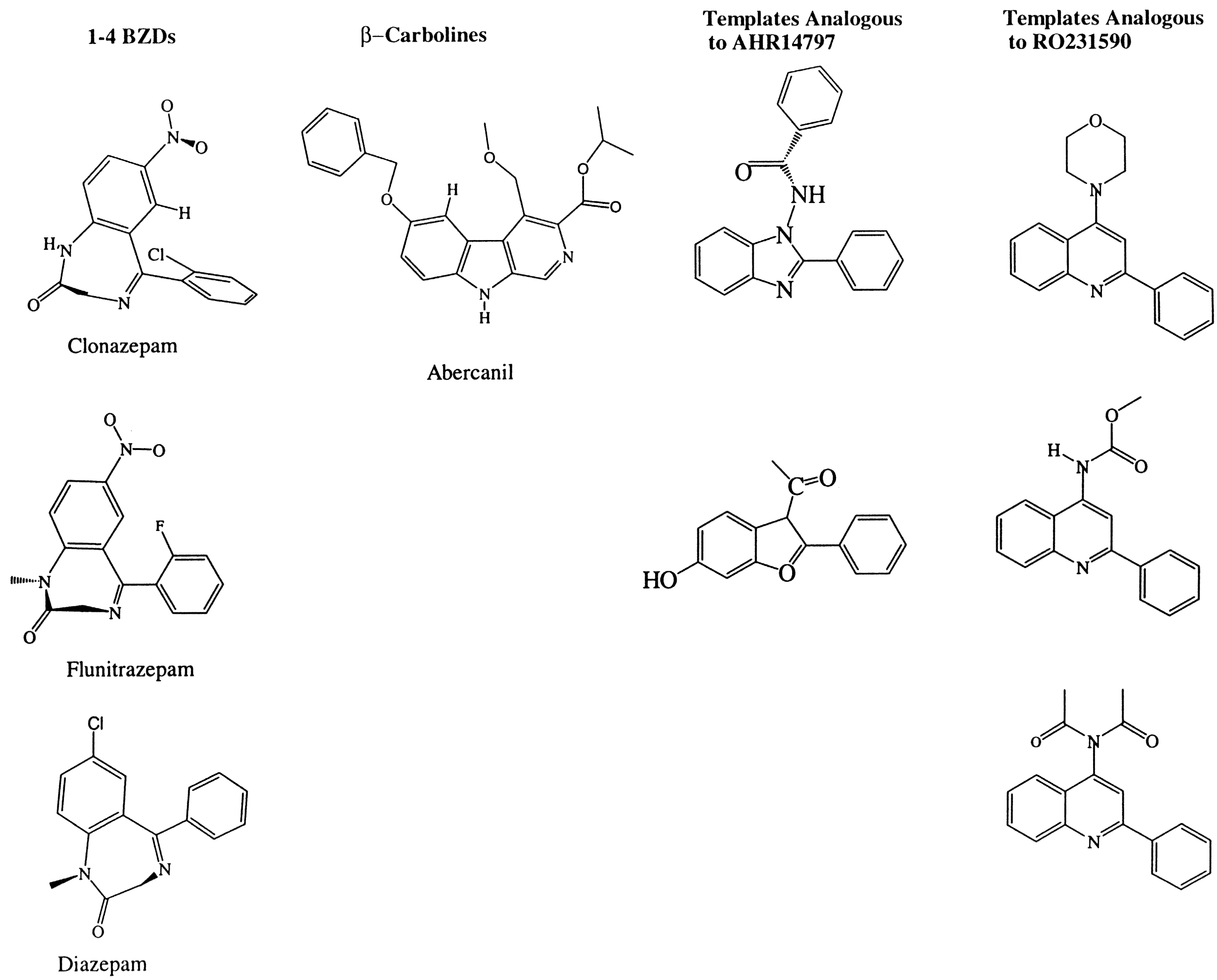

Fig. 6. A small sampling of compounds obtained from database searches based on the current pharmacophore.

ligands. In addition to finding compounds within the training set, such as flunitrazepam and abercanil, additional high efficacy sedatives were found such as clonazepam and diazepam providing additional validation. Perhaps, more important are the novel compounds shown in Fig. 6. These compounds have templates analogous to the RO23-1590 and AHR 14749 compounds found to show some discrimination between anxiolysis and sedation.

The fourth assessment is that the 3D pharmacophore for these compounds including agonists, inverse agonists and antagonists at the sedation endpoint has predictive ability in retrieval of known sedative compounds in chemical databases. The development of a pharmacophore which identifies properties common to the compounds which have any one of these three types of activities and the demonstration that these are absent in those that have no effect provides support for 3D recognition pharmacophore development based on in vivo behavioral data. This result also provides additional support that the binding pocket of agonists, inverse agonists and antagonists are overlapping.

The fifth type of assessment is based on the recent study examining the effects of diazepam in H101R-mutated $\alpha 1$ subunit in mice that definitively indicates a role of the $\alpha 1$ subunit in $GABA_A$/benzodiazepine receptor sedative

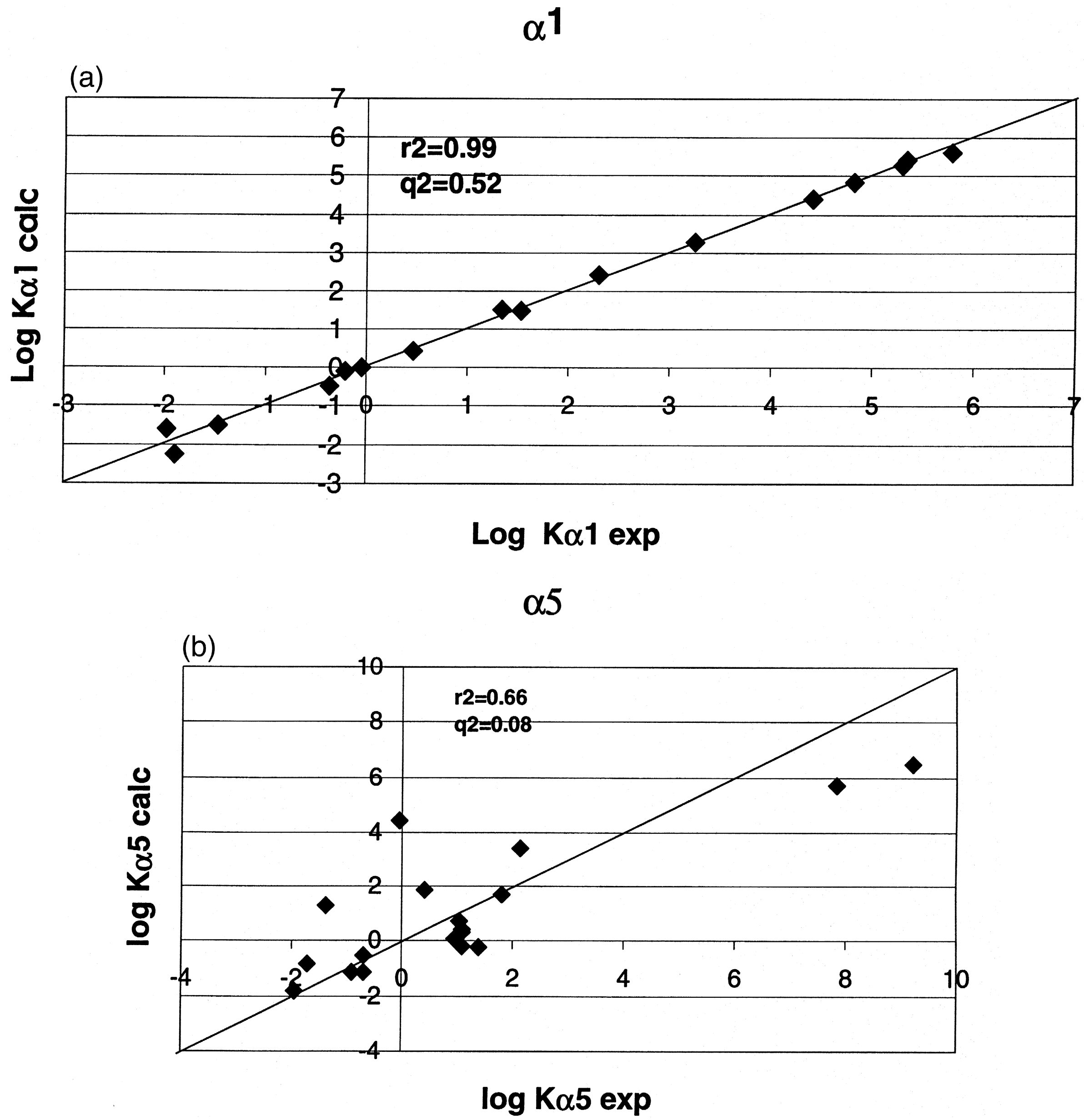

Fig. 7. (a) CoMSIA (3D-QSAR) for correlation of predicted binding to benzodiazepine receptor subtypes containing $\alpha 1$ subunits. (b) CoMSIA (3D-QSAR) for correlation of predicted binding to benzodiazepine receptor subtypes containing $\alpha 5$ subunits.

response. The question addressed was to what degree does the 3D recognition pharmacophore developed from such in vivo data also indicate high affinity and strong correlations for binding to $GABA_A$ benzodiazepine receptors containing $\alpha 1$ subunits.

Fig. 7a shows the result of a PLS analysis for the comparison of the computed binding based on the pharmacophore with experimentally determined $\alpha 1$ binding affinities for the training set based on the CoMSIA method. The correlation with $\alpha 1$ binding affinities is quite good in several respects. The cross-validated r^2 value (q^2) is 0.5 with an optimal number of components of 4. In order for such a 3D-QSAR to be reasonably predictive the analysis should give q^2 values of greater than 0.3 (the 95% confidence interval for q^2). For such diverse template training sets a q^2 value of ca. 0.5 is in fact quite good and is likely to be quite predictive. A non cross-validated r^2 of 0.99 was obtained with a standard error estimate of 0.2. The overall results indicate the 3D-QSAR model based on the MOLMOD pharmacophore to be a promising model for binding to $\alpha 1$ containing benzodiazepine receptor subtypes.

For the sake of comparison, an analogous comparative molecular similarity index analysis using the molecular overlap generated in the sedation recognition pharmacophore analysis but now using $\alpha 5$ binding data for the compounds in the training set. This is a negative control since $\alpha 5$ containing receptors have been shown not to lead to activity at the sedation endpoint. Consistent with this facet, as shown in Fig. 7b, an attempt to derive a 3D-QSAR model based on the sedation pharmacophore shows very poor correlation with $\alpha 5$ binding data with a small q^2 value, indicating almost no predictivity for binding to $\alpha 5$ containing $GABA_A$ benzodiazepine receptors. The results thus show a linkage between the 3D pharmacophore for recognition of receptors leading to activity at the sedation endpoint and high affinity for the compounds used to determine this pharmacophore at benzodiazepine receptors containing $\alpha 1$ subunits.

4. Discussion

A 3D pharmacophore has been determined that embodies the common molecular determinants of ligand recognition of $GABA_A$/benzodiazepine receptors that elicit sedation effects. Recognition of these receptors is associated with compounds with two hydrogen bonding groups on opposite sides of the molecular axis defined by a hydrophobic moiety on one end and the LUMO ring on the other end of the benzodiazepine receptor ligand. The pharmacophore obtained, shown in Fig. 2, provides by complementarity, insight into the types of ligand receptor interactions important for recognition and the spatial arrangements of these complementary receptor residues. The long axis is defined by the distance between the hydrophobic moiety postulated to interact with hydrophobic residues in the receptor and the aromatic ring that could serve as an electron acceptor from a corresponding amino acid electron donor. A ring with polar moieties is between these two anchoring points. It is postulated to have both hydrophobic and electrostatic interactions with a corresponding ring of a receptor residue such as tyrosine or tryptophan that could have both hydrophobic and electrostatic components. The two proton accepting moieties of the ligand are on opposite sides of this long axis and postulated to interact with corresponding proton donor residues, such as arginine, lysine, serine or threonine in the receptor. A 3D-QSAR analysis based on the 3D pharmacophore deduced was shown to give good correlation with binding affinities of ligands to $GABA_A$ benzodiazepine receptor subtypes containing $\alpha 1$ subunits, but not to benzodiazepine receptor subtypes containing $\alpha 5$ subunits. Both of these computational results are consistent with experiment.

In this work, we have used a training set with abundant molecular diversity, carefully characterized behavioral data and a template free method of extracting the 3D determinants of recognition of receptors initiating activity at the sedation endpoint. These same methods will be used to develop determinants of recognition of receptor subtypes associated with other behavioral endpoints. In prior studies in this laboratory, the effect of a set of structurally diverse benzodiazepine receptor ligands was assessed at five different behavioral endpoints: anxiolysis, sedation, hyperphagia, hypothermia, and anticonvulsant activity (Davies et al., 1994; Chen et al., 1996). The striking observation was made that many of the compounds evaluated exhibited behavioral heterogeneity. That is, the same compound could produce qualitatively different effects, namely agonist, inverse agonist, antagonist or no effect at different behavioral endpoints. There were no compounds evaluated that were without effect at all five behavioral endpoints assessed. This observation rules out lack of transport across the blood brain barrier as the origin of the lack of effect of a given compound at a specific endpoint. Furthermore, the pattern of heterogeneity in these studies was not the same for each compound. These results provide further evidence for $GABA_A$/benzodiazepine receptor heterogeneity and that a different, if perhaps overlapping, set of functional $GABA_A$ receptor benzodiazepine subtypes, are responsible for initiating a given behavioral response.

The validated 3D pharmacophore determined from this data will be used as the basis for rationale computer aided discovery of candidate benzodiazepine receptor ligands with more selective sedation activity. This effort is of clinical importance since benzodiazepines remain the mainstay of therapy for insomnia (Wagner et al., 1998) with the benzodiazepine ligand zolpidem, the most commonly used hypnotic in the U.S. (Lobo and Greene, 1997). In mammals, sedation can be induced by the enhancement of $GABA_A$ receptor function via the benzodiazepine-binding site (Luddens and Korpi, 1995). Despite benzodiazepines relative safety, they are associated with several adverse effects, seen more often with high doses and long-term use (Wagner et al., 1998). These effects include residual daytime sedation ''hangover'', rebound insomnia and anterograde amnesia. Additional long-term use can lead to tolerance with withdrawal symptoms appearing if abrupt discontinuation occurs. Clearly, it is desirable to ascertain the structural features associated with selective sedation response with elimination or minimization of adverse side effects.

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